N'-NITROSONORNICOTINE (NNN)

This substance was considered by a previous Working Group, in October 1977 (IARC, 1978). Since that time, new data have become available, and these have been incorporated into the monograph and taken into account in the present evaluation.

1. Chemical and Physical Data

1.1 Synonyms and trade names

Chem. Abstr. Services Reg. Nos: 80508-23-2; 16543-55-8[1]; 84237-38-7[2]

Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-,(S)-[1]; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-, (+,-)-[2]

IUPAC Systematic Name: 1'-Demethyl-1'-nitrosonicotine

Synonyms: 1'-Demethyl-1'-nitrosonicotine; 1'-desmethyl-1'-nitrosonicotine; 1'-nitroso-1'-demethylnicotine; *N*-nitrosonornicotine; 1'-nitrosonornicotine; nitrosonornicotine; 1-nitroso-2-(3-pyridyl)pyrrolidine; 3-(1-nitroso-2-pyrrolidinyl)pyridine

1.2 Structural and molecular formulae and molecular weight

N
N=O
N

$C_9H_{11}N_3O$ Mol. wt: 177.2

1.3 Chemical and physical properties of the pure substance

(*a*) *Description*: Yellow oil that solidifies on standing in the cold

(*b*) *Boiling-point*: 154°C at 0.2 mm (Hu *et al.*, 1974)

[1]The Chemical Abstracts Services Registry Number and Name refer to a single stereoisomer (S).
[2]The Chemical Abstracts Services Registry Number and Name refer to the racemic mixture that was synthesized and used in the biological studies reported in this monograph.

(*c*) *Melting-point*: 47°C

(*d*) *Spectroscopy data*: Mass (Hecht *et al.*, 1981a), ultraviolet, infrared, and nuclear magnetic resonance spectra (Hu *et al.*, 1974) have been reported.

(*e*) *Reactivity*: Can be reduced to the corresponding hydrazine with lithium aluminium hydride (Neurath & Duenger, 1966). For formation of *N'*-nitrosonornicotine-1-*N*-oxide, see Hecht *et al.* (1980a). For other reactions, see Chen *et al.* (1979) and Hecht *et al.* (1979).

1.4 Technical products and impurities

NNN is not produced commercially.

2. Production, Use, Occurrence and Analysis

2.1 Production and use

NNN was prepared by Boyland *et al.* (1964) by treating nornicotine with sodium nitrite in dilute hydrochloric acid. Using a variation of this procedure, Hu *et al.* (1974) obtained a yield of 93%. It has also been made by the reaction of nicotine-*N'*-oxide with nitrites or nitrogen dioxide (Klimisch & Stadler, 1976).

When nicotine was reacted with five equivalents of sodium nitrite in aqueous solution at 90°C for 3 h at pH 3.4-4.2, NNN was the identified reaction-product formed in the second highest yield (8.8% of the starting nicotine) (Hecht *et al.*, 1978a).

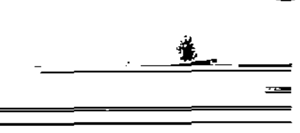
No evidence was found that NNN has ever been produced in commercial quantities or that it has any uses other than as a laboratory chemical.

2.2 Occurrence

(*a*) *Tobacco and tobacco smoke*

NNN has not been detected in freshly harvested tobacco (Hecht *et al.*, 1978a); however, it was detected in freshly homogenized, leaf-cured tobacco (Andersen *et al.*, 1982).

NNN has been reported to be produced by nitrosation of nicotine, the most prevalent alkaloid in commercial tobacco, and only to a minor extent from the tobacco alkaloid, nornicotine, during the curing, ageing, processing and smoking of tobacco (Hecht *et al.*, 1978b). NNN has been found in tobacco at levels of 0.2-130 mg/kg, in snuff products at 0.8-77 mg/kg, in chewing tobacco at 1.0-90.6 mg/kg, and in cigarette smoke (mainstream) at 0.1-3.7 μg/cigarette (Hoffmann *et al.*, 1974; Klus & Kuhn, 1975; Hoffmann *et al.*, 1976; Munson & Abdine, 1977; Hoffmann *et al.*, 1979; US Department of Health and Human Services, 1981; Andersen *et al.*, 1982; Brunnemann *et al.*, 1983a; Chamberlain & Arrendale, 1983; Hoffmann & Hecht, 1983). Additional quantities of NNN may be formed in the oral cavity during oral use of snuff or tobacco (Hoffmann *et al.*, 1980a; Hoffmann & Adams, 1981; Brunnemann *et al.*, 1983a; Sipahimalani *et al.*, 1984).



Two studies reported no correlation between nicotine content and NNN levels in 12 flue-cured tobacco samples (Brunnemann *et al.*, 1983b; Chamberlain & Arrendale, 1983).

Table 1 summarizes the data obtained from analyses of smoke from selected commercial cigarettes with and without filter tips and shows the effectiveness of the filters of cigarettes D and E in decreasing the occurrence levels of NNN (Hoffmann *et al.*, 1982).

Table 1. Reduction of nicotine and NNN concentrations in cigarette smoke by filtration[a]

Cigarette	Lengthsmoked/ length of cigarette (mm)	Nicotine (mg/cigarette)[b]	NNN (μg/cigarette)[b]
A NF	50/65	1.8	0.8
F	50/85	1.1 (-40%)	0.5 (-44%)
B NF	50/65	1.8	0.5
F	50/85	1.3 (-29%)	0.3 (-39%)
C NF	50/65	1.7	0.8
F	50/85	1.2 (-30%)	0.5 (-38%)
D NF	50/65	1.6	1.1
F	50/85	1.1 (-34%)	0.3 (-69%)
E[c] NF	50/73	1.6	1.8
F	50/100	1.1 (-34%)	0.5 (-71%)

[a]Data from Hoffmann *et al.* (1982)
[b]In parentheses, percentage changes in yields on comparing filtered (F) and non-filtered (NF) smoke
[c]Perforated filter tip

Nicotine and NNN concentrations found in cigarette and cigar tobaccos, in both their mainstream (during puff drawing) and sidestream (generated during smouldering of tobacco in between puffs) smoke, and in pipe and chewing tobacco are presented in Table 2.

In studies with [2'-^{14}C]NNN, the NNN in tobacco smoke was demonstrated to be partially formed during smoking; however, 40-50% were shown to originate from NNN in the tobacco by direct transfer into the mainstream smoke. Approximately 46% of the NNN in the smoke of a US, blended, non-filter cigarette originated from the tobacco, whereas the rest was formed during smoking. In the case of a French, dark-tobacco cigarette with alkaline smoke, the transfer of NNN from the tobacco into the smoke was about 41%; therefore, the balance of 59% was formed during smoking (Hoffmann *et al.*, 1980b; Adams *et al.*, 1983b).

NNN concentrations found after analysis of snuff obtained in Sweden, Denmark, Federal Republic of Germany and the USA were higher than those in cigarette tobacco presented above. The results are summarized in Table 3. NNN concentrations differed not only among snuff brands but also in samples of the same brand bought in different cities. The latter differences were attributed to possible variations in NNN content between batches and/or effects of ageing. The effects of ageing were demonstrated by opening individual portions (packed in aluminium foil) and storing the snuff in the open air: within eight days, the NNN content had increased by 34% and then remained stable. The data presented in Table 3 suggest that part of the nitrate in the snuff (present at about 2%) was reduced to nitrite while standing in the open air, an occurrence that was not observed in a germ-free atmosphere (Hoffmann & Adams, 1981; Hoffmann *et al.*, 1982).

Addition of nitrate to cigarettes increased the yields of nitrosamines in mainstream smoke (Adams *et al.*, 1984). This may be important, because the levels of nitrate in cigarettes have increased from about 0.5% to 1.2-1.5% over the last 20 years (US Department of Health and Human Services, 1982).

Table 2. NNN concentrations in cigarette, cigar, pipe and chewing tobacco, and cigarette and cigar mainstream and sidestream smoke[a]

Tobacco product[b]	NNN concentration		
	In tobacco (mg/kg)[c]	In mainstream smoke (μg/cig)	In sidestream smoke (μg/cig)
Burley cigarette, NF	7.0	3.7	6.1
Bright cigarette, NF	0.2	0.6	1.7
Commercial cigarette, NF	1.7	0.2	1.7
Commercial cigarette, FA	1.4	0.3	0.2
US cigarettes[d]			
F	2.6	–	–
F	2.2	–	–
NF	1.8	–	–
NF, 70 mm	2.0	–	–
F, menthol	1.9	–	–
F, light	4.4	–	–
F, ultra-light	3.2	–	–
US cigarette, NF[e]	–	1.0	–
Kentucky 1R1[d]	0.7	–	–
Kentucky 1R1, NF	0.6	0.4	0.2
German (Federal Republic) cigarettes[f]			
Brand A, NF	–	0.5	–
Brand B, NF	–	0.2	–
Brand C, FA	–	0.2	–
Brand D, FA	–	0.1	–
Brand E, FA	–	0.2	–
Brand F, FA	–	0.2	–
Commercial French cigarette, NF, 70 mm	2.9	0.5	–
Commercial French cigarettes,			
FA, 70 mm	2.7	0.5	–
NF	11.9	3.2	–
FA	11.9	0.0	–
FP	11.9	0.7	–
British cigarette, NF[g]	0.3	–	–
Little cigar, FA	45.0	5.5	0.9
Cigar (Columbian tobacco) (5.7 g)	10.7	3.2	16.6
Cigar[d]	2.9	–	–
Loose-leaf tobacco[d]	1.2	–	–
Fine-cut chewing tobacco	39.0	NA[h]	NA
Fine-cut chewing tobacco[e]	45.6	NA	NA
Japanese tobaccos[i]			
Cigarette 1	1.1	–	–
Cigarette 2	D	–	–
Cigarette 3	D	–	–
Cigarette 4	D	–	–
Cigarette 5	D	–	–
Cigarette 6	ND	–	–
Kiseru tobacco	1.8	–	–
Pipe tobacco 1	D	–	–
Pipe tobacco 2	ND	–	–
Pipe tobacco 3	ND	–	–
Chewing tobacco[g]			
Scrap-leaf A	3.5	NA	NA
Scrap-leaf B	3.9	NA	NA
Scrap-leaf C	8.2	NA	NA
Plug A	3.4	NA	NA
Plug B	4.3	NA	NA
Fine-cut	90.6	NA	NA
Indian tobacco for betel quid	2.4	NA	NA
Pipe tobaccos[j]			
US	1.6	-	-
US	3.1	-	-

[a]Data from Hoffmann *et al.* (1980a), unless otherwise noted
[b]All cigarettes and the little cigar were 85 mm long, unless otherwise noted. Abbreviations: NF, non-filter; FA, cellulose acetate filter; FP, paper filter; F, unspecified filter
[c]Abbreviations: D, presence doubtful; ND, not detected (detection limit, 0.01 mg/kg (Hecht *et al.*, 1975a))
[d]Data from Brunnemann *et al.* (1983b); products (excluding Kentucky 1R1) were purchased in Westchester County, NY, in 1982
[e]Data from Adams *et al.* (1983a); cigarettes and tobacco used were purchased in Westchester County, NY, in 1981
[f]Data from Rühl *et al.* (1980); cigarettes were popular brands purchased in Berlin in 1979
[g]Data from Hoffmann *et al.* (1976)
[h]NA, not applicable
[i]Data from Bharadwaj *et al.* (1975)
[j]Data from Munson and Abdine (1977)

Table 3. Nicotine, nornicotine and NNN concentrations in commercial snuff[a]

Snuff origin	Type of packaging[b]	Nicotine (g/kg)	Nornicotine (g/kg)	NNN (mg/kg)[c]
USA				
Brand I New York and Tennessee	A	23.8	0.6	39
Brand II New York and Tennessee	A	23.4	0.5	26.5
Brand III New York and Tennessee	B	14.5	0.3	3.5
Federal Republic of Germany				
Brand I Munich	C	5.7	0.8	6.7
Brand II Munich	C	5.4	0.6	6.1
Sweden				
Brand I Umeå	A	15.2	0.3	8.6
Brand I Uppsala	A	15.0	0.3	6.1
Brand I Lund	A	15.1	0.3	8.9
Brand II Umeå	A	18.1	0.2	8.4
Brand II Uppsala	A	18.0	0.2	5.2
Brand II Lund	A	18.1	0.2	10.3
Brand III Umeå	A	6.0	0.05	7.8
Brand III Uppsala	A	6.2	0.05	3.5
Brand III Lund	A	6.7	0.05	3.8
Brand IV Umeå	A	11.2	0.07	9.7
Brand IV Uppsala	A	11.2	0.06	77.1
Brand IV Lund	A	11.4	0.08	8.2
Brand V Umeå	D	21.7	0.9	4.7
Denmark (3)				
Brand I Copenhagen	E	11.1	0.8	6.4
Brand II Copenhagen	E	21.2	0.9	4.5
Brand III Copenhagen	E	30.5	0.6	8.0
Ageing test[d]: 0 time	–	–	–	4.7
8 days	–	–	–	6.3
Snuff and smokeless tobacco[e]				
US	–	–	–	3.2
US	–	–	–	9.3

[a]Data from Hoffmann and Adams (1981), unless otherwise noted; values are given for dry snuff, moisture content about 50%

[b]Abbreviations: A, waxed-paper container with metallic lid, containing approximately 50 g; B, 25 individual portions of approximately 11 g packaged in paper in a plastic container; C, plastic foil-lined aluminium bags containing 100 g; D, individual snuff portion in a paper bag packaged in a crimped airtight aluminium envelope, with 10 envelopes in a plastic bag amounting to approximately 10 g; E, hard-plastic container

[c]NNN values are averages of three runs.

[d]Data from Hoffmann *et al.* (1982). The aluminium foil-wrapped package (Swedish brand V) was opened at '0 time'.

[e]Data from Munson and Abdine (1977)

(b) Human tissues and secretions

Formation of additional quantities of NNN by the reaction of salivary nitrite with nicotine or nornicotine during oral use of snuff or tobacco chewing has been implied from in-vitro studies (Hoffmann & Adams, 1981). Incubation of fine-cut chewing tobacco (for 3 h at 37°C) with saliva resulted in a value for NNN of 127 µg/g, an increase of 44% over the 88.6 µg/g found in the original chewing tobacco. A similar value, 133 µg/g, was obtained when fine-cut chewing tobacco was extracted with saliva for 18 h at 20°C (Hecht *et al.*, 1975a).

Saliva was examined from women who had been long-term oral-snuff users and who were employed in two southern US furniture companies. The wide range in NNN concentrations (5.0-125 ng/g) in the saliva of individual users indicated that during oral use of snuff NNN is extracted from the tobacco plug at varying rates (Hoffmann & Adams, 1981).

In another study, saliva of four women who were long-term (>10 years) oral-snuff users was analysed on two different days after they had used a specific brand of snuff with known concentrations of NNN. The results (Table 4) indicate that the saliva of users contains significant amounts of NNN and that the levels vary significantly between subjects, as well as between samples from the same individual taken on different days. The variations in NNN values were at least partially explained by differences in the intensity with which individuals practised the habit at different times, and perhaps also by varying rates of salivation (Hoffmann & Adams, 1981; Hoffmann *et al.*, 1982).

Table 4. Nicotine, nornicotine and NNN concentrations in snuff and in the saliva of women who were long-term oral-snuff users[a]

Subject	Age (years)	Snuff			Saliva[b]		
		Nicotine (mg/g)	Nor-nicotine (mg/g)	NNN (μg/g)	Day of sampling	Nicotine (mg/g)	NNN (μg/g)
1	41	23.4	0.05	26.5	1	0.2	0.2
					2	0.5	0.1
2	37	23.4	0.05	26.5	1	0.07	0.03
					2	0.4	0.1
3	44	23.4	0.05	23.1	1	1.2	0.4
					2	1.6	0.3
4	52	23.6	0.06	24.8	1	0.2	0.03
					2	0.4	0.06

[a]Data from Hoffmann and Adams (1981)

[b]Saliva of three women who did not use snuff (controls) was free of nicotine and tobacco-specific *N*-nitrosamines.

NNN was not detected in the combined gastric juices of 14 volunteers who each smoked 28 cigarettes per day (Schweinsberg *et al.*, 1975). [The Working Group noted that analytical methods have improved since 1975-1977.]

NNN was found in the saliva of chewers of betel quid with tobacco at levels of 1.2-38 ng/g [mean, 13.1 ng/g] (Wenke *et al.*, 1984) and 1.6-14.7 ng/ml (mean, 7.5 ng/ml); it was also detected in the saliva of chewers of tobacco, at levels of 16.5-59.7 ng/ml (mean, 33.4 ng/ml) (Nair *et al.*, 1985).

2.3 Analysis

Standard methods for the analysis of NNN are described in detail in an IARC manual on selected methods of analysis (Egan *et al.*, 1983).

3. Biological Data Relevant to the Evaluation of Carcinogenic Risk to Humans

3.1 Carcinogenicity studies in animals

(*a*) *Oral administration*

Rat: A group of 20 male Fischer rats, seven weeks old, was given 200 mg/l NNN (purity, >99%; Hu *et al.*, 1974) in the drinking-water on five days per week for 30 weeks (esti-

mated total dose, 630 mg). Animals were killed when moribund or after 11 months, and all animals, except those lost by cannibalism or autolysis, were necropsied. All 12 rats necropsied from the treated groups had developed oesophageal tumours (11 papillomas and three carcinomas); in addition, one pharyngeal papilloma and three carcinomas of the nasal cavity with invasion of the brain were observed. No tumour was observed in 19 untreated controls (Hecht *et al.*, 1975b; Hoffmann *et al.*, 1975).

A group of 15 female Sprague-Dawley rats, eight weeks old, was given approximately 7 mg NNN [purification and characterization checked by ultraviolet and mass spectra] per rat per day in the drinking-water (354 mg/l) on five days per week for 44 weeks. All rats died within 46 weeks, and all had nasal-cavity adenocarcinomas. In addition, one squamous-cell papilloma of the oesophagus, one squamous-cell papilloma of the forestomach and one hepatocellular tumour occurred in three rats surviving 43 weeks or longer (Singer & Taylor, 1976). [The Working Group noted that no detail concerning parallel or historical control groups was reported.]

Groups of 12 male and 12 female Fischer 344 rats, six to eight weeks of age, received 0.012% (w/v) NNN (purity, >99%; Hu *et al.*, 1974) in the drinking-water continuously for 36 weeks (total doses, approximately 3.6 and 3.3 mmol, respectively). The combined incidence of oesophageal tumours in animals of both sexes, including squamous-cell carcinomas (six) and papillomas, was 23/24 rats by 11 months; all rats had died by month 12. Nasal-cavity tumours, including 15 benign and 15 malignant tumours, occurred in 21/24 rats. One female rat had a tracheal papilloma. No such tumour was reported in 12 male or 12 female controls (Hecht *et al.*, 1983a).

Two groups of 30 male Fischer 344 rats were pair-fed either a control liquid diet or an isocaloric liquid diet containing ethanol (6% w/v) starting at nine weeks of age. Starting at 13 weeks of age, rats were placed on control or ethanol liquid diets containing 17.5 mg/l NNN (purity, 98%) continuously, seven days per week for the following 27 weeks until each animal had consumed 1 mmol NNN (3.2-3.7 mmol/kg bw). Thereafter, the liquid diets were replaced by a standard diet, and rats were maintained until killed when moribund or when 98 weeks old (mean survival time, 83 weeks). Two control groups consisted of 26 rats receiving liquid diet with or without ethanol and receiving three injections per week of 0.9% saline solution [number of injections not specified, presumably 56-66]. At the end of the series of injections, the control rats were switched to standard diet and maintained and killed in parallel to the treated rats. All of the NNN-treated rats developed head-and-neck tumours (nasal cavity, oesophagus and tongue), whereas none of the controls had a tumour at these sites. In the group receiving NNN and ethanol, there were 20 benign tumours [not histologically classified], one aesthesioneuroepithelioma, three squamous-cell carcinomas and two anaplastic tumours of the nasal cavity; 13 benign tumours [not histologically classified]; seven squamous-cell carcinomas of the oesophagus; three benign tumours of the root of the tongue; and three adenocarcinomas of the lung. In the group receiving NNN but no ethanol, there were 11 benign tumours, two aesthesioneuroepitheliomas and five squamous-cell carcinomas of the nasal cavity; 16 benign tumours and nine squamous-cell carcinomas of the oesophagus; two benign tumours of the root of the tongue; and one squamous-cell carcinoma of the lung. Among 26 control rats given neither ethanol nor NNN there was one lung adenocarcinoma. Among 26 control rats given ethanol, there was one adenoma and one adenocarcinoma of the lung. Each group had a number of other benign or malignant tumours distributed among several tissues and organs, as would be expected in ageing rats (Castonguay *et al.*, 1984).

Hamster: Groups of 10 male and 10 female Syrian golden hamsters, six to seven weeks of age, received 0.016% (w/v) NNN (purity, >99%; Hu *et al.*, 1974) in the drinking-water con-

tinuously for 31 weeks (total doses, approximately 1.9 and 2.8 mmol). The experiment was terminated after 96 weeks. Papillomas of the nasal cavity developed in 4/20 animals and papillomas of the trachea in 2/20; one lymphoma of the caecum and one angiosarcoma of the liver also occurred. No such tumour occurred in 10 male or 10 female controls (Hecht *et al.*, 1983a).

(*b*) *Skin application*

Mouse: A group of 20 female Ha/ICR/mil Swiss mice, seven to eight weeks of age, received thrice-weekly applications of 0.1 ml of a 0.03% solution of NNN (purity, >99%; Hu *et al.*, 1974) in acetone for 50 weeks. No skin tumour developed in this group (Hoffmann *et al.*, 1976). [The Working Group noted that no data were available on tumours in other organs.]

(*c*) *Subcutaneous and/or intramuscular administration*

Rat: Groups of 12 male and 12 female Fischer 344 rats, seven weeks of age, received thrice-weekly s.c. injections of 10 mg [0.06 mmol] NNN (purity, >99%) in 0.3 ml trioctanoin for 20 weeks (total dose, 600 mg; 3.4 mmol). Animals were maintained until they died spontaneously or were killed after 12 months. Malignant tumours of the nasal cavity (15 olfactory neuroblastomas and two rhabdomyosarcomas) developed in 17/24 rats; in addition, 3/24 rats developed benign nasal-cavity tumours [this category included papillomas, adenomatous polyps and villous polyps, but the histological types of these three tumours were not stated explicitly], and another developed a lesion described as a neoplastic nodule of the liver. No such tumour was seen in 24 vehicle controls that received trioctanoin only (Hecht *et al.*, 1980b).

Three groups of 15-27 male and 15-27 female Fischer 344 rats, nine weeks of age, received thrice-weekly s.c. injections of NNN (purity, >99%; Hu *et al.*, 1974) in trioctanoin (total doses, 9.0, 3.0 or 1.0 mmol/kg bw), or trioctanoin alone (vehicle controls; 52 animals) for 20 weeks. Animals were killed when moribund or when only 20% of rats in a group were still alive. No animal was still alive after 60, 120 or 130 weeks in the three groups, respectively; no difference in body weight was observed between treated and control animals. Nasal-cavity tumours were found in 12/14 males and 15/15 females given the high dose; all were malignant aesthesioneuroepitheliomas, squamous-cell carcinomas, anaplastic carcinomas or spindle-cell sarcomas. In the medium-dose group, 8/15 males and 5/15 females had malignant nasal-cavity tumours, and 3/15 males and 4/15 females had benign nasal-cavity tumours (squamous-cell papillomas, transitional-cell papillomas or polyps). In the low-dose group, 4/27 males had malignant, and 11/27 males and 12/27 females had benign nasal-cavity tumours. Benign oesophageal tumours [histological type not reported] were seen in 4/14 males and 3/15 females in the high-dose group, 5/15 males and 2/15 females in the medium-dose group, and 1/27 males and 1/27 females in the low-dose group. In the three groups combined, four rats developed benign liver tumours, four, bladder tumours, one, a lung adenocarcinoma and 11, lung adenomas. The occurrence of nasal-cavity tumours suggests a dose-response relationship. Four benign liver tumours were observed in controls treated with trioctanoin only (Hoffmann *et al.*, 1984).

Two groups of 30 male Fischer 344 rats were pair-fed either a control liquid diet or an isocaloric liquid diet containing ethanol (6% w/v) starting at nine weeks of age. Starting at 13 weeks of age, each animal was injected s.c. with 10 mg/kg bw NNN (purity, 98%) in a 0.9% saline solution, three times per week until a total dose of 1 mmol/rat (3.2-3.7 mmol/kg;

56-66 injections) had been delivered. The liquid diets were replaced by a standard diet 24 h after the last injection. Rats were killed when moribund or when 98 weeks old (mean survival time, 83 weeks). Two control groups maintained on liquid diet with or without ethanol received s.c. injections of 0.9% saline solution without NNN [number of injections not specified, presumably 56-66] and were then switched to standard diet. Of the NNN-treated rats, 22/30 on the diet with ethanol and 26/30 on the diet without ethanol had head-and-neck tumours, whereas none were seen in controls with or without ethanol in the diet. In the group receiving NNN and ethanol, there were two benign tumours [not histologically classified], 17 aesthesioneuroepitheliomas, two squamous-cell carcinomas and one sarcoma of the nasal cavity; two benign tumours [not histologically classified] and one squamous-cell carcinoma of the oesophagus; and one benign tumour of the root of the tongue. No lung tumour was observed. In the group given NNN but not ethanol, there were four benign tumours, 18 aesthesioneuroepitheliomas, one squamous-cell carcinoma and one anaplastic tumour of the nasal cavity; no oesophageal or tongue tumour; and one lung adenoma. Among 26 control rats given neither ethanol nor NNN, there was one lung adenocarcinoma. Among 26 control rats given ethanol, there was one adenoma and one adenocarcinoma of the lung. Each group had a number of other benign or malignant tumours distributed among several tissues and organs, as would be expected in ageing rats (Castonguay *et al.*, 1984).

Groups of 15 male and 15 female Fischer 344 rats, six weeks of age, were given s.c. injections of 10 mg NNN, 2',5',5'-trideutero-NNN or 2'-deutero-NNN (purity, >99%; Chen *et al.*, 1979) in saline three times per week for 14 weeks (total of 41 injections; total dose, 410 mg/rat). All surviving animals were killed at 21 months. Controls received s.c. injections of saline alone. The mean length of survival was 13 months for the animals treated with NNN and 15 months for animals treated with the deuterated compounds. A total of 20 animals treated with NNN (males and females combined) had invasive olfactory tumours [unspecified]; the corresponding numbers for animals treated with 2',5',5'-trideutero-NNN and 2'-deutero-NNN were 15 and 13; no such tumour was seen in controls. No significant increase in the incidence of tumours at other sites was observed (Hecht *et al.*, 1982a).

Hamster: Groups of 10 male and 10 female Syrian golden hamsters, eight to 10 weeks of age, received thrice-weekly s.c. injections of 5 mg NNN (purity, >99%; Hu *et al.*, 1974) in saline for 25 weeks (total dose, 375 mg). A group of 10 males and 10 females received injections of saline only and served as vehicle controls. Animals were killed when moribund, or at termination of the experiment at 83 weeks. Of 19 effective animals, 12 developed single papillary tumours of the trachea within 83 weeks (first tumour after 38 weeks). One animal had an adenocarcinoma of the nasal cavity after 45 weeks. No such tumour was observed in 17 effective controls given saline only (Hilfrich *et al.*, 1977).

A group of 15 male and 15 female Syrian golden hamsters, eight to 10 weeks of age, received 19 s.c. injections of 8.5 mg (0.048 mmol) NNN (purity, >99%) in 0.3 ml trioctanoin on a three-injection-per-week schedule (total dose, 160 mg [0.91 mmol]). The experiment was terminated after 16 months. Survival rates were the same in treated and untreated animals. Among the 28 effective animals in the treated group, five tracheal papillomas, one lung adenoma and one tumour described as an 'undifferentiated carcinoma of the leg' were reported. A further group of 10 males and 10 females received thrice-weekly s.c. injections of 2.5 mg (0.012 mmol) NNN in trioctanoin for 25 weeks. The experiment was terminated after 17 months; after 13 months, 60% of the treated animals were still alive. Among the 18 effective animals, one tracheal papilloma and one adenocarcinoma of the lung were reported. No respiratory-tract tumour was observed in 30 vehicle controls receiving trioctanoin only (Hoffmann *et al.*, 1981).

(d) Intraperitoneal administration

Mouse: Groups of 20 male and 20 female Chester Beatty stock mice, approximately six weeks old, were injected i.p. once a week with 0.1 ml NNN [purity unspecified] dissolved in arachis oil (2%) for 41 weeks; 14 males and 11 females died during the first seven months with no tumour. Of eight animals that died after the eighth month, seven (five females and two males) had multiple pulmonary adenomas. Groups of 15 male and 15 female mice injected i.p. weekly with arachis oil served as vehicle controls; the only tumour reported among these 30 control mice was a single lung adenoma in a mouse killed at 11 months (Boyland *et al.*, 1964). These results were confirmed in A/HE mice (Hoffmann *et al.*, 1976).

In a screening assay for potential carcinogenicity using pulmonary adenomas as an end-point in strain A mice, a group of 21 female strain A/J mice, six to eight weeks old, received thrice-weekly i.p. injections of 1 mg NNN (purity, >99%; Hu *et al.*, 1974) in 0.2 ml saline for seven weeks (total of 22 injections; total dose, 22 mg [0.13 mmol]) and were held without further treatment for an additional 30 weeks. A further 23 mice received NNN in trioctanoin by the same schedule; and an untreated control group of 25 mice was available. Among the 23 mice that received NNN in trioctanoin, 12 had lung adenomas, one had a lung adenocarcinoma, one had an undifferentiated carcinoma of the salivary glands and one had a malignant lymphoma. Of the 21 mice that received NNN in saline, 16 had lung adenomas and one had an undifferentiated carcinoma of the salivary gland. In untreated, saline and trioctanoin controls, lung adenomas were seen in 1/25, 3/25 and 5/24 mice, respectively (Hecht *et al.*, 1978b).

In another study, female A/J mice, six to eight weeks old, received 22 thrice-weekly i.p. injections of NNN (purity, >99%; Hu *et al.*, 1974) or 2',5',5'-trideutero-NNN (purity, >99%; Chen *et al.*, 1979) in saline over seven weeks (total dose, 0.12 mmol). Animals were killed 30 weeks after the last injection. Of the treated mice, 16/24 animals developed lung tumours, compared with 7/24 controls receiving a saline solution. The total number of lung tumours in NNN-treated animals was 29, 10 of which were malignant, compared with nine (one malignant) in controls. No tumour was found in other organs. Of mice treated with 2',5',5'-trideutero-NNN, 20/25 had 37 lung tumours, 14 of which were malignant (Castonguay *et al.*, 1983a).

Hamster: Groups of 21 male Syrian golden hamsters received thrice-weekly i.p. injections of NNN (purity, >99%; Hu *et al.*, 1974) in saline for 25 weeks beginning at 13 weeks of age [total dose, 1 mmol (low-dose) or 2 mmol (high-dose)]. The animals were maintained on a liquid diet with or without ethanol, beginning at eight weeks of age. Of the 21 animals in the low-dose group receiving an ethanol-free diet, one developed an invasive nasal-cavity tumour (reported to be of olfactory origin but not further classified) and four developed tracheal papillomas. Of the high-dose group receiving an ethanol-free diet, 5/21 developed nasal-cavity tumours (olfactory but not further classified; two invasive), and 9/21 developed tracheal papillomas. A number of other tumours were observed; many of these were similar to those seen in vehicle controls, except for an adenosquamous-cell carcinoma of the lung. Ethanol did not appear to influence the tumorigenicity of NNN in this study (McCoy *et al.*, 1981a).

(e) Carcinogenicity of metabolites

In a screening assay for potential carcinogenicity using pulmonary adenomas as an end-point in strain A mice, groups of 25 female A/J mice, six to eight weeks old, received thrice-weekly i.p. injections of 0.2 ml 3'-hydroxy-NNN, 4'-hydroxy-NNN or NNN-1-*N*-oxide

(purity, >99%; Hecht *et al.*, 1980a) in saline for seven weeks (22 injections; total dose, 0.12 mmol) and were killed 30 weeks later. Twenty-five animals served as untreated controls, and 24 vehicle controls received saline only by the same schedule. Ten untreated controls had a total of 16 lung tumours, two of which were carcinomas [histological type not specified]; the average multiplicity of lung tumours per mouse was 0.6 ± 0.9. Seven vehicle controls had a total of nine lung tumours, one of which was malignant; the average multiplicity was 0.4 ± 0.6 per mouse. Of mice treated with 3'-hydroxy-NNN, 12/25 had a total of 23 lung tumours, including six carcinomas, and 0.9 ± 1.4 lung tumours per mouse. In mice treated with 4'-hydroxy-NNN, 19/25 had lung tumours, with a total of 41 lung tumours, including 13 carcinomas, and an average multiplicity of 1.6 ± 1.5 lung tumours per mouse. Of 25 mice treated with NNN-1-*N*-oxide, 16 had a total of 21 lung tumours, of which six were carcinomas; the average multiplicity was 0.8 ± 0.7 lung tumours per mouse. Tumours of other organs included a gastric papilloma in a mouse treated with 3'-hydroxy-NNN and an angioma of the adrenal medulla in a mouse treated with NNN-1-*N*-oxide (Castonguay *et al.*, 1983a).

Rat: A group of 12 male and 12 female Fischer 344 rats, six to eight weeks of age, was given 0.012% (w/v) NNN-1-*N*-oxide (purity, >99%; Hecht *et al.*, 1980) in the drinking-water (0.012%, w/v) every day for 36 weeks; the experiment was terminated after 104 weeks. Oesophageal papillomas developed in 5/12 males and 0/12 females; squamous-cell carcinomas of the oesophagus developed in 3/12 males and 3/12 females. Nasal-cavity tumours (papillomas and/or carcinomas) were found in 11/12 males and 7/12 females. In addition, two pulmonary adenomas and two papillomas of the tongue developed in treated males. No such tumour was found in 12 male or 12 female control rats (Hecht *et al.*, 1983a).

Hamster: Groups of 10 male and 10 female Syrian golden hamsters, six to seven weeks of age, were given NNN-1-*N*-oxide in the drinking-water (0.016%, w/v) daily for 31 weeks; the experiment was terminated after 96 weeks. No tumour of the nasal cavity or trachea was reported (Hecht *et al.*, 1983a).

3.2 Other relevant biological data

(a) *Experimental systems*

Toxic effects

The subcutaneous LD_{50} of NNN in male rats observed for eight days was >1000 mg/kg bw. In rats that died, haemorrhages were observed in the lungs and abdominal organs and epithelial-cell necrosis in the posterior nasal cavities and liver (Hoffmann *et al.*, 1975).

Effects on reproduction and prenatal toxicity

No data were available to the Working Group.

Absorption, distribution, excretion and metabolism

Single intravenous doses of 3.4-7 mg/kg bw [2'-^{14}C]NNN were distributed within 1-5 min throughout the tissues of C57Bl mice, as determined by whole-body autoradiography. After 24 h, non-extractable radioactivity was present in the tracheobronchial and nasal mucosa, liver, submaxillary and sublingual salivary glands, and oesophagus. Binding to the melanin of the eyes and hair was observed *in vivo* and *in vitro* (Brittebo & Tjälve, 1980; Waddell &

Marlowe, 1980, 1983). Single intravenous doses of 4-5 mg/kg bw [2'-^{14}C]NNN were distributed in tissues of Fischer 344 or Sprague-Dawley rats within 5 min of injection. A high uptake of radioactivity was seen in the mucosa of the ethmo-, naso- and maxilloturbinates, in the submaxillary salivary glands, lachrymal glands, Zymbal glands, tarsal glands of the eyelids, preputial glands, oesophagus and tongue, and in the contents of the stomach. After 24 h, non-extractable radioactivity was present in the nasal, tracheobronchial and oesophageal mucosa, and in the liver (Brittebo & Tjälve, 1981).

Male Fischer 344 rats that received a subcutaneous injection of 3-300 mg/kg bw [2'-^{14}C]NNN excreted 73-91% of the dose in urine over 48 h. Less than 1% of the dose was detected in expired air (Chen *et al.*, 1978; Hecht *et al.*, 1981b). Male Syrian golden hamsters that were given subcutaneous injections of 60 mg/kg bw [2'-^{14}C]NNN excreted 62-78% of the dose in urine over 48 h, 10% in faeces and <0.5% in expired air as $^{14}CO_2$ (Hoffmann *et al.*, 1981). Urine was also the major pathway of excretion in male A/J mice after intraperitoneal injection of 50 mg/kg bw [2'-^{14}C]NNN (Hecht *et al.*, 1981b).

Metabolic pathways of NNN are summarized in Figure 1. The metabolites formed initially result from hydroxylation of each position of the pyrrolidine ring, giving compounds *2-5*, and from oxidation of the pyridine nitrogen, yielding compound *1*.

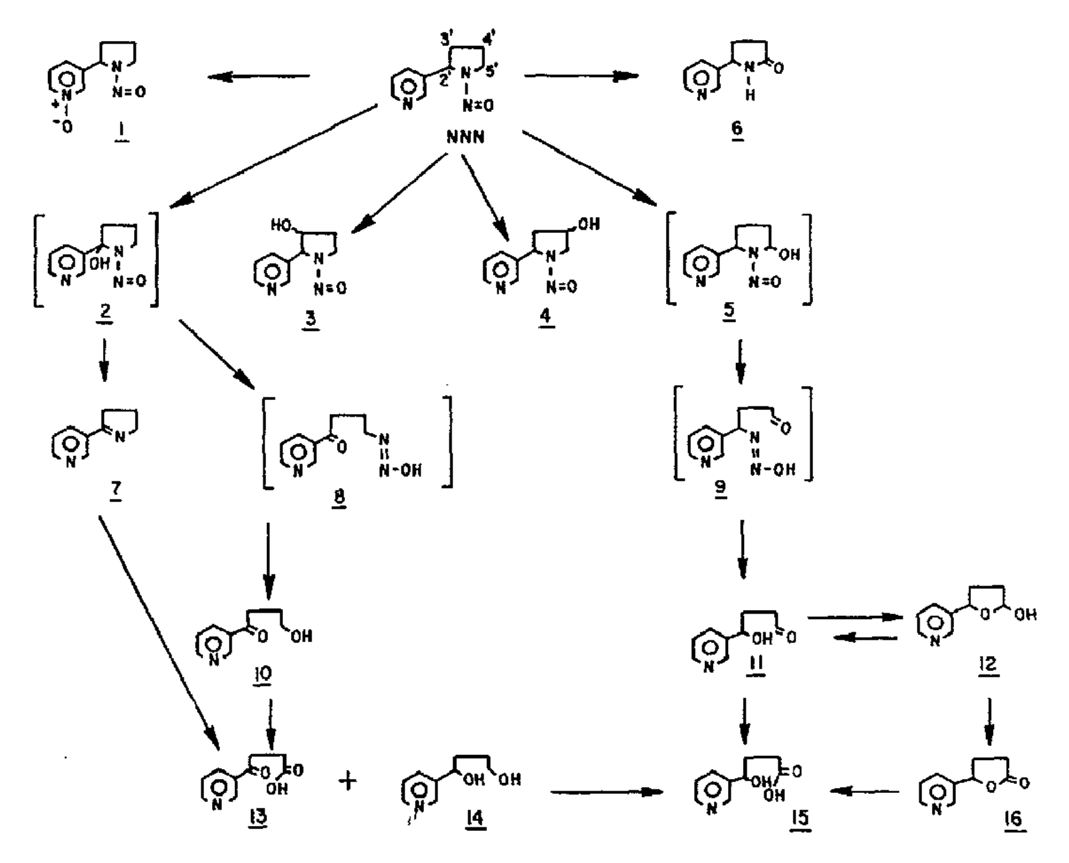
2'-Hydroxylation and 5'-hydroxylation are thought to be the major activation processes in NNN metabolism. 2'-Hydroxylation gives 2'-hydroxy-NNN (*2*), which is unstable and tautomerizes spontaneously to the electrophilic intermediate *8*. This intermediate, which is also formed from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (see monograph, p. 209), induces mutations in *Salmonella typhimurium* (Chen *et al.*, 1978; Hecht *et al.*, 1983b). It also reacts with water to give 4-hydroxy-1-(3-pyridyl)-1-butanone (*10*), a major metabolite of NNN formed by rat-liver microsomes (Chen *et al.*, 1978, 1979). Metabolite *10* is further oxidized *in vivo* to give 4-oxo-4-(3-pyridyl)butyric acid (*13*), a major urinary metabolite of NNN in rats, hamsters and mice (Chen *et al.*, 1978). It accounted for 13-31% of a dose of NNN administered to Fischer 344 rats (Hecht *et al.*, 1981b) and 15% of one given to Syrian golden hamsters (Hoffmann *et al.*, 1981).

3'-Hydroxylation and 4'-hydroxylation of NNN lead to the formation of the stable metabolites, 3'- and 4'-hydroxy-NNN (*3* and *4*), which have been detected as minor urinary metabolites in Fischer 344 rats and as minor products of metabolism by Fischer 344 rat-liver microsomes (Hecht *et al.*, 1980a).

5'-Hydroxylation yields 5'-hydroxy-NNN (*5*), which is unstable and tautomerizes spontaneously to the electrophile *9*. Since 5'-hydroxy-NNN is unstable, the corresponding acetate was tested, and was found to induce mutations in *S. typhimurium* (Chen *et al.*, 1978). Reaction of *9* with water gives 2-hydroxy-5-(3-pyridyl)tetrahydrofuran (*12*), a major metabolite of NNN formed by rat-liver microsomes (Chen *et al.*, 1978). Oxidation and ring-opening of this metabolite *in vivo* yield 4-hydroxy-4-(3-pyridyl)butyric acid (*15*), the principal urinary metabolite of NNN in rats (37-53% of the dose administered), hamsters (39% of the dose) and mice (Chen *et al.*, 1978; Hecht *et al.*, 1981b; Hoffmann *et al.*, 1981).

Pyridine-*N*-oxidation of NNN gives NNN-1-*N*-oxide (*1*), a stable metabolite formed by Fischer 344 rat-liver microsomes and excreted in the urine of Fischer 344 rats (7-11% of the dose administered) and Syrian golden hamsters (3% of the dose) (Hecht *et al.*, 1980a, 1981b; Hoffmann *et al.*, 1981). Another urinary metabolite of NNN, norcotinine (*6*), constituted 3-5% of a dose administered to rats (Hecht *et al.*, 1981b).

Figure 1. Metabolism of NNN. Structures in brackets represent hypothetical intermediates[a]



[a]From Hecht *et al.* (1981b). Compounds: *1*, *N'*-nitrosonornicotine-1-*N*-oxide (NNN-1-*N*-oxide); *3*, 3'-hydroxy-*N'*-nitrosonornicotine (3'-hydroxy-NNN); *4*, 4'-hydroxy-*N'*-nitrosonornicotine (4'-hydroxy-NNN); *6*, norcotinine; *7*, myosmine; *10*, 4-hydroxy-1-(3-pyridyl)-1-butanone; *11*, 4-hydroxy-1-(3-pyridyl)butanal; *12*, 2-hydroxy-5-(3-pyridyl)tetrahydrofuran; *13*, 4-oxo-4-(3-pyridyl)butyric acid; *14*, 4-hydroxy-4-(3-pyridyl)-1-butanol; *15*, 4-hydroxy-4-(3-pyridyl)butyric acid; *16*, 5-(3-pyridyl)tetrahydrofuran-2-one

Metabolism of NNN by 2'- and 5'-hydroxylation has been demonstrated in various animal tissues. Cultured Fischer 344 rat oesophagus and nasal mucosa, target tissues in which NNN induces tumours, metabolized NNN extensively, with preferential 2'-hydroxylation; the 2'- to 5'-hydroxylation ratios after 24 h were 3.4 and 1.8, respectively, as compared to a ratio of 0.3 in cultured Syrian golden hamster oesophagus, a non-target tissue (Hecht *et al.*, 1982b; Brittebo *et al.*, 1983). Pieces of rat nasal mucosa converted [2'-^{14}C]NNN to tissue-bound metabolites more efficiently than either rat liver or oesophagus (Brittebo & Tjälve, 1981). In corroboration of these findings, Sprague-Dawley rats that received two intravenous injections of 0.18 mg/kg bw [2'-^{14}C]NNN had greater tissue-bound radioactivity levels

in the nasal mucosa than in any other tissue one, 4 and 24 h after administration. Labelling of DNA was detected in the liver and in nasal mucosa (Löfberg *et al.*, 1982). Cultured A/J mouse peripheral lung principally metabolized NNN by 2'- and 5'-hydroxylation, giving a ratio of 2'- to 5'-hydroxylation of 0.6 24 h after administration (Castonguay *et al.*, 1983a).

The levels of 2'- and 5'-hydroxylation of NNN in various tissues are affected by inducers of the cytochrome-P-450 mixed-function oxidase system. Pretreatment of Fischer 344 rats with 500 mg/kg bw Aroclor 1254 four days prior to killing resulted in a 20-fold induction of 2'-hydroxylation and a 1.9-fold induction of 5'-hydroxylation in hepatic microsomes (Chen *et al.*, 1979). Pretreatment of Fischer 344 rats with 80 mg/kg bw per day phenobarbital intraperitonally for four days prior to killing increased hepatic microsomal 2'-hydroxylation 1.5-fold but had no effect on 5'-hydroxylation; pretreatment of Syrian golden hamsters by the same protocol had no effect on 2'-hydroxylation but increased 5'-hydroxylation 2.5-fold; pretreatment of Fischer 344 rats with 20 mg/kg bw per day 3-methylcholanthrene intraperitoneally for four days prior to killing increased 2'-hydroxylation 2.7-fold but caused a 2.2-fold decrease in 5'-hydroxylation in hepatic microsomes; no effect was observed in hamsters (McCoy *et al.*, 1981b).

Administration of a liquid diet, in which ethanol isocalorically replaced carbohydrate, to Syrian golden hamsters for four weeks resulted in a 1.8-fold increase in liver microsomal 5'-hydroxylation but did not affect 2'-hydroxylation. No effect of ethanol on NNN metabolism was observed in cultured tracheal rings (Chen *et al.*, 1980; McCoy *et al.*, 1982). Treatment of male Fischer 344 rats with a liquid diet containing ethanol for four weeks caused a 1.5-fold increase in 2'-hydroxylation and a 1.7-fold increase in 5'-hydroxylation in cultured nasal mucosa; no effect was observed in cultured lingual mucosa or oesophagus (Castonguay *et al.*, 1984). Pretreatment of male Fischer 344 rats with a variety of isothiocyanates and related compounds, either by gavage 2 h prior to killing or in the diet for two weeks prior to killing, generally caused an inhibition of 2'- and 5'-hydroxylation in cultured rat oesophagus (Chung *et al.*, 1984).

Mutagenicity and other short-term tests

In the presence of a liver microsomal preparation from Aroclor-induced rats, NNN (highest dose tested, 2.5 μmol/plate) caused a dose-dependent increase in mutations in *Salmonella typhimurium* TA100 (Bartsch *et al.*, 1980) and induced mutations in strain TA1530 at 1000 μg/plate (5.7 μmol/plate) (Andrews *et al.*, 1978).

NNN (10^{-3} and 10^{-2}M) induced unscheduled DNA synthesis in freshly isolated hepatocytes from adult rats (Williams & Laspia, 1979).

(*b*) *Humans*

No data were available to the Working Group on toxic effects or on effects on reproduction and prenatal toxicity.

Absorption, distribution, excretion and metabolism

Human liver microsomes obtained from biopsies catalysed 2'- and 5'-hydroxylation of NNN and gave a 2'- to 5'-hydroxylation ratio of 0.6 (Hecht *et al.*, 1979). Human tissues obtained at immediate autopsy and cultured for 24 h with [2'-^{14}C]NNN metabolized NNN to compounds *1* and *15* (see Fig. 1) by *N*-oxidation and 5'-hydroxylation, respectively, as follows (values in nmol/100 μg DNA): buccal mucosa, 0.2 ± 0.2 and 0.03 ± 0.03; trachea, 0.7

± 0.7 and 0.2 ± 0.1; oesophagus, 0.2 ± 0.2 and 0.4 ± 0.9; bronchus, 0.8 ± 1.1 and 0.9 ± 1.7; peripheral lung, 0.5 ± 0.2 and 0.4 ± 0.7; urinary bladder, 2.4 ± 3.4 and 1.1 ± 1.8. Metabolite *13* (see Fig. 1), formed by 2'-hydroxylation, was detected in only a few explants. The ratio of compounds *1* to *15* was different in human tissues from that in the corresponding tissues of animals (Castonguay *et al.*, 1983b).

Mutagenicity and chromosomal effects

No data were available to the Working Group.

3.3 Case reports and epidemiological studies of carcinogenicity to humans

No data were available to the Working Group.

4. Summary of Data Reported and Evaluation

4.1 Exposure data

N'-Nitrosonornicotine (NNN) has been found in a variety of tobacco products (chewing tobacco, snuff, cigarettes and cigars), in mainstream and sidestream smoke from cigars and cigarettes, in saliva of chewers of betel quid with tobacco and in saliva of oral-snuff users. Some of the NNN in saliva appears to be formed endogenously from nitrite in saliva and tobacco alkaloids. Thus, there is widespread exposure to NNN among users of tobacco products and those exposed to sidestream smoke.

4.2 Experimental data

NNN was tested for carcinogenicity in rats, mice and hamsters by different routes of administration in multiple experiments. Following its oral administration, NNN produced carcinomas of the upper digestive tract, mainly the oesophagus, and of the nasal cavity in rats and nasal-cavity tumours in hamsters. Following its subcutaneous administration, NNN produced primarily tumours of the nasal cavity in rats and tumours of the trachea in hamsters. Intraperitoneal injection produced lung tumours in mice and tumours of the nasal cavity and trachea in hamsters. There was evidence of a dose-response relationship after subcutaneous administration of NNN to rats.

Several metabolites of NNN were tested in mice by intraperitoneal injection, producing lung tumours. NNN-1-*N*-oxide was also tested in rats and hamsters by oral administration; it produced nasal-cavity and oesophageal tumours in rats.

NNN is mutagenic to *Salmonella typhimurium* in the presence of an exogenous metabolic system. It induces unscheduled DNA synthesis in primary cultures of rat hepatocytes.

4.3 Human data

No case report or epidemiological study of the carcinogenicity of NNN was available to the Working Group.

Overall assessment of data from short-term tests: *N'*-Nitrosonornicotine[a]

	Genetic activity			Cell transformation
	DNA damage	Mutation	Chromosomal effects	
Prokaryotes		+		
Fungi/green plants				
Insects				
Mammalian cells (*in vitro*)	+			
Mammals (*in vivo*)				
Humans (*in vivo*)				
Degree of evidence in short-term tests for genetic activity: *Limited*				Cell transformation: No data

[a]The groups into which the table is divided and '+' are defined on pp. 16-17 of the Preamble; the degrees of evidence are defined on p. 18.

4.4 Evaluation[1]

There is *sufficient evidence*[2] for the carcinogenicity of *N'*-nitrosonornicotine to experimental animals.

No data on humans were available.

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[1]For description of the italicized term, see Preamble, pp. 15-16.

[2]In the absence of adequate data on humans, it is reasonable, for practical purposes, to regard chemicals for which there is *sufficient evidence* of carcinogenicity in animals, as if they presented a carcinogenic risk to humans.

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